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Abstract

I**ntroduction:** Skewed or non-random X-chromosome inactivation may be more common in women with epithelial ovarian cancer and early-onset breast cancer. We tested this hypothesis in a group of 235 breast cancer cases and 253 controls (mean age 45.8 years, s.e. 0.25) from a larger population-based case-control study conducted in Poland.

Methods: We measured X-chromosome inactivation in lymphocyte DNA digested with the methylation-specific enzyme *HpaII* using an assay for the polymorphic trinucleotide repeats in the *AR* gene. We considered a sample as skewed using an adjusted measure (relative to the undigested sample) with a cut-point of 75%, and an unadjusted measure where skewed was defined as more than 90% of the signal from one allele in the *HpaII* digested sample. Odds ratios (OR) and 95% confidence intervals (CI) estimated with logistic regression models were used as a measure of risk for categorical variables. We also used non-parametric Wilcoxon rank-sum and median statistics to compare continuous measures of skewing.

Results: There were no significant differences in any of the skewing measures between cases and controls, both within the entire sample set and among the 360 pre-menopausal women below age 50. Using the adjusted skewing measure among pre-menopausal subjects under age 50, 14% of cases versus 11% of controls were skewed, OR = 1.2, 95% CI 0.6 – 2.3; using the unadjusted measure, OR = 0.9, 95% CI 0.4 – 2.0.

Conclusions: While we cannot rule out a subtle difference of approximately 2-fold or less, we have failed to find a significant difference in the prevalence of skewed X-chromosome inactivation in younger women with breast cancer compared to controls.

Random X-chromosome inactivation in female embryos results in roughly equal expression of transcripts from maternally and paternally derived X-chromosomes.[1] Skewed (or non-random) X-chromosome inactivation is present if most, or all, of the transcripts are derived from only one of the parental X-chromosomes.[2, 3] Two case-control studies have suggested that skewed X-chromosome inactivation, measured in lymphocytes, is more common in ovarian cancer and early-onset breast cancer cases than in controls.[4, 5] Skewed X-chromosome inactivation may play a role in carcinogenesis if, for example, the preferentially expressed chromosome contains mutations or polymorphisms in cancer-related genes. In addition, the recent observation of the involvement of the BRCA1 protein in normal X-chromosome inactivation further highlights the possible involvement of X-chromosome inactivation in breast cancer.[6] We studied whether skewed X-chromosome inactivation was more common in early-onset breast cancer cases from a population-based case-control study conducted in Poland.

METHODS

Subjects

Subjects were drawn from a population-based case-control study of 2,386 breast cancer cases and 2,503 controls aged 19–74 years residing in Warsaw and Łódź, Poland. This study was approved by Institutional Review Boards at the Cancer Center and M. Sklodowska-Curie Institute of Oncology, Warsaw and Nofer Institute of Occupational Medicine, Lodz in Poland and at the U.S. National Cancer Institute, National Institutes of Health. Cases were newly diagnosed with pathologically confirmed *in situ* or invasive breast cancer between February 1, 2000 and January 31, 2003, and controls were randomly selected from population lists, frequency matched on city of residence and age in 5-year categories. (Garcia-Closas, under submission & [7]) Response rates for the personal interview were 79% for cases and 69% for controls and of those interviewed, 84% of cases and 92% of controls gave a blood sample. Blood samples were collected on average 38 days after diagnosis. DNA was extracted from buffy coats using the automated PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN). The target subject group for the present analysis, selected in August, 2003, included those who reported that they were still having menstrual periods (i.e, pre-menopausal subjects) for whom DNA was already available. Cases included only those with invasive breast

cancer not known to have had chemotherapy before sample collection. We identified 644 subjects meeting these criteria.

Because laboratory analyses were begun before data collection was completed, 59 subjects analyzed for skewed X-chromosome inactivation were later determined to be postmenopausal or had received chemotherapy prior to blood collection. Subject recruitment has since been completed, and the 280 premenopausal women with invasive breast cancer and no history of chemotherapy included in this analysis represents 67% of all potentially eligible subjects. Sixty-five subjects were homozygous at the androgen receptor (*AR*) locus (see laboratory methods below), 79 samples did not amplify reliably, and two subjects gave discordant results, leaving 235 cases and 253 controls analyzed for skewed X-chromosome inactivation.

Laboratory Methods

A trinucleotide-repeat in the *AR* gene on the X-chromosome is highly polymorphic, methylation at two CpG sites near the repeat correlates with silencing, and this locus has been used in numerous studies of skewed X-chromosome inactivation.[2, 8-10] Each DNA sample is amplified twice: once after it is digested with a methylation-specific restriction enzyme (*HpaII*) and once after it is digested with an "irrelevant" enzyme (*Rsa I)*, that does not cut within the *AR* amplicon (herein referred to as "undigested"). Primer sequences were as previously described[9] except that the forward primer was labeled with 6-FAM and products were analyzed on an ABI 3730 and GeneMapper software (Applied Biosystems, Foster City, CA). We switched polymerase enzymes from Taq Gold (Applied Biosystems, Foster City, CA) to Herculase (Stratagene, La Jolla, CA) because it resulted in peaks with heights that were more similar in the undigested state. We calculated the proportion of the signal derived from the shorter (allele 1) peak as {allele 1 peak height/(allele 1 peak height + allele 2 peak height)}. When the difference in length between the two alleles was one repeat unit (3 base pairs), we adjusted the peak height by 27% owing to the influence of stutter bands.

Statistical Analyses

Skewing is not an all-or-nothing phenomenon but continuous measures of skewing were not normally distributed, nor were various transformations of the data. We therefore evaluated

skewing with two non-parametric measures, the Wilcoxon two-sample rank-sum test (Z statistic with continuity correction of 0.5, two-tailed) and the median two-sample test (Z statistic, two-tailed). We also categorized subjects as skewed using both "adjusted" and "unadjusted" measures at various cut-points. Because in the undigested state the peak heights were not always equal, we devised an "adjusted" skewing measure by using the allele 1 peak proportion in the undigested sample as a baseline. For example, if the allele 1 peak proportion was 40% in the undigested sample, the allele 1 proportion could increase 60 percentage points (to 100%) or decrease 40 (to 0%). If the allele 1 peak proportion was 70% in the digested sample, the % skewing was calculated as 50% (i.e., 30 percentage points of a potential 60), and if it was 10% in the digested sample, the % skewing was 75% (30/40). Samples were attempted at least three times and 195 samples were scored twice. For subjects with two readings, the % skewed was averaged between the two runs. Those with <50%, 50-74%, and $\geq$ 75% skewing were categorized as normal, partially skewed, and skewed. In keeping with several prior publications, we also used an "unadjusted" measure by considering only the peak heights in the *HpaII* restriction enzyme digested sample, categorizing samples as skewed if more than 90% of the signal proportion was from one allele. Odds ratios were calculated using logistic regression, adjusting for age, age at menarche, number of full-term births, age at first full-term birth, menopausal status (when appropriate), and first-degree family history of breast cancer. For the 195 subjects with two adjusted skewing measurements, the intraclass correlation coefficient[11] was calculated with the macro INTRACC (http://ftp.sas.com/techsup/download/stat/intracc.html). All statistical analyses were performed with SAS 9.1 (SAS Institute, Cary, NC).

RESULTS

The subset of cases and controls available for this analysis, performed before subject recruitment was completed, were well-matched to the entire case group with regard to area of residence and age (the two matching variables for the study overall), and those subjects that could be analyzed for skewed X-chromosome inactivation (because they were heterozygous for the *AR* polymorphism) did not differ from the entire case group (Table 1). Among 195 subjects with two laboratory skewing determinations, the intraclass correlation coefficient was 0.93. The distribution of the adjusted and unadjusted skewing measures among all cases and controls is shown in Figure 1. Non-parametric analyses did not reveal a significant difference in skewing

between cases and controls (Table 2). Using the percent skewed measure ("adjusted" for the allele proportion in the undigested sample), *P* values were 0.4 and 0.8 for the Wilcoxon rank-sum and median test statistics in the entire sample and 0.3 for both statistics in pre-menopausal subjects below age 50. Using the unadjusted measure of the proportion of signal from one allele in the restriction enzyme digested sample only, *P* values were 0.7 and 0.9 for the Wilcoxon rank-sum and median test statistics in entire sample, and 0.7 and 0.8 for in pre-menopausal subjects below age 50.

Logistic regression analyses, comparing the proportion skewed (calculated with both the adjusted and unadjusted measures) between cases and controls, similarly did not show a significant difference (Table 2). Among all analyzable subjects, slightly more cases (14.9%) than controls (11.9%) were skewed using the "adjusted" measure with a cut-point of 75%, but this difference was not statistically significant, adjusted OR = 1.2, 95% C.I. 0.7 – 2.1. Among pre-menopausal women below age 50 there was no significant difference either, adjusted OR = 1.2, 95% C.I. 0.6 – 2.3. The unadjusted measure of skewing was not significantly different either, 9.1% in all cases versus 10.2% in controls, adjusted OR = 1.1, 95% C.I. 0.6 – 2.0. Among pre-menopausal women below age 50, the proportion skewed using the unadjusted measure was nearly identical. Analyses stratified by family history of breast cancer were conflicting between the adjusted and unadjusted skewing measures: the adjusted skewing measure was somewhat lower in family history positive subjects compared to those without a family history (9% vs 15% in cases and 8% vs 12% in controls), while for the unadjusted measure, family history positive cases and controls had higher skewing (14% vs 10% in cases and 15% vs 9% in controls). These analyses, however, were based on only 22 and 13 family history positive subjects in the cases and controls, respectively.

DISCUSSION

We have studied younger women with breast cancer from a large population-based case-control study and have not found a significant difference in the proportion showing skewed X-chromosome inactivation using an androgen receptor (*AR*) gene assay in lymphocyte DNA. This is in contrast to a report of a higher prevalence of skewed X-chromosome inactivation in a study of early-onset breast cancer from Sweden,[5] and to the initial report of higher prevalences in

women with epithelial ovarian cancer.[4] Our study is the largest to date, and while we cannot rule out more subtle differences between cases and controls, the upper confidence intervals on our odds ratios were all 2.3 or less.

The hypothesis that skewed X-chromosome inactivation may play a role in breast and ovarian cancer etiology was initially generated from a study of 174 informative women with epithelial ovarian cancer and 45 controls without ovarian cancer (Table 3).[4] That study used the same *AR* PCR amplicon and defined "skewed" as those where the ratio of peak heights for the two alleles (determined visually) was ≥ 3 in the *Hha I* restriction enzyme digested lymphocyte DNA sample. Cases were much more likely to be skewed (53%) compared to controls (33%), unadjusted OR = 2.6. They also noted that 9 of 11 *BRCA1* mutation carriers among the cases were skewed. This observation heightened our interest in the subsequent basic research finding that the BRCA1 protein is involved in normal embryonic X-chromosome inactivation.[6] A small study of breast cancer cases and controls from Sweden also found higher rates of skewed X-chromosome inactivation in the cases.[5] They defined "skewed" as those demonstrating 90% or more of the signal from one allele in the *HpaII* restriction enzyme digested lymphocyte DNA samples using the *AR* assay. In contrast to older patients, 13% of 40 cases diagnosed at age 45 or younger were skewed compared to 1% of 95 controls, OR = 13.

There is no clear standard for categorizing a sample as showing skewed (non-random) X-chromosome inactivation. A commonly used assay is the one used in this study in which the highly polymorphic trinucleotide repeat within exon 1 of the *AR* gene is amplified in a PCR reaction using DNA that is pre-digested with *HpaII* (or *HhaI*). These enzymes do not cut DNA if there is methylation of the cytosines in the two recognition sequences within this PCR amplicon. If X-chromosome inactivation is random, the peak heights representing the two chromosomes (the two alleles) will be nearly equal (owing to equal methylation and therefore equal restriction digestion of two alleles).Departures from equal peak heights indicates varying degrees of inactivation. Ideally this departure from equal allele peak heights would be treated as a continuous variable and subjected to analysis of variance, but numerous means of quantifying this skewing, along with numeric transformations, were not normally distributed. This requires one to use potentially less powerful non-parametric tests and to arbitrarily determine cut-points at which samples are categorized as skewed or not. Particularly because the relative peak heights

of the two alleles were not equal even in assays of the DNA processed with mock restriction digests ("undigested" DNA), we devised the adjusted skewing measure before linking the laboratory and epidemiologic variables. To facilitate comparison with most prior publications, we also categorized samples using only the information in the restriction-digested peak heights.

Upon inspection of the frequency histograms for both adjusted and unadjusted skewing measures (Figure 1), one gets a hint of more cases at the very extreme of the tail. None of our analyses using numerous cut-points for the various measures calculated before inspection of these figures, however, showed a significant difference between cases and controls, including for the smaller group of women age 45 and younger (data not shown.) This is not to say that there are no possible cut-points that might result in a "post-hoc" comparison with a P-value less than 0.05. Non-parametric tests similarly did not confirm a significant difference. We designed this study to have at least 80% power to detect a two-fold increase in risk, assuming 15% of controls would show skewing (alpha=.05). Using our adjusted skewing measure, approximately 12% of controls showed skewed X-chromosome inactivation, which would have reduced our power very slightly. However, despite numerous outcome measures and statistical tests, we did not observe a significant difference in the rates of skewed X-chromosome inactivation, and the upper confidence intervals around our odds ratios were generally 2.0 to 2.3. Our data suggests that skewed X-chromosome inactivation, as measured in peripheral lymphocytes using the *AR* gene assay, is not more common in younger women with breast cancer compared to controls.

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Figure Legends

Figure 1

(A) Distribution of adjusted skewing measure according to case-control status.
Frequency histogram showing the number of subjects in each 5 percentage point interval of the adjusted skewing measure (% skewed from baseline, range 0% to 100%). Controls are indicated by solid bars; breast cancer cases by light bars.

(B) Distribution of unadjusted skewing measure according to case-control status.
Frequency histogram showing the number of subjects in each 2.5 percentage point interval of the adjusted skewing measure (% signal from one allele in restriction enzyme digested sample, range 50% to 100%). Controls are indicated by solid bars; breast cancer cases by light bars.

Table 1. Characteristics of study subjects in the X-chromosome inactivation study in Polish Breast Cancer Study

	Eligible subjects (N=644)				Subjects analyzed for skewing (N=488)			
	Cases (N=314)		Controls (N=330)		Cases (N=235)		Controls (N = 253)	
Characteristic	N	%	N	%	N	%	N	%
Study site								
Warsaw	184	59%	215	65%	143	61%	157	62%
Łódź	130	41%	115	35%	92	39%	96	38%
Age								
25-35	8	3%	10	3%	7	3%	7	3%
35-39	26	8%	26	8%	22	9%	21	8%
40-44	71	23%	78	24%	58	25%	61	24%
45-49	127	40%	148	45%	92	39%	111	44%
50-54	64	20%	54	16%	45	19%	39	15%
55-65	18	6%	14	4%	11	5%	14	6%
Age at menarche								
< 12	109	35%	76	23%	86	37%	57	23%
13	70	23%	78	24%	46	20%	63	25%
14	80	26%	104	32%	61	27%	82	33%
15	26	8%	31	10%	16	7%	22	9%
≥ 16	24	8%	36	11%	21	9%	24	10%
missing	5		5		5		5	
No. of full-term births								
nulliparous	39	12%	33	10%	34	14%	25	10%
1	106	34%	113	34%	79	34%	91	36%
2	134	43%	138	42%	97	41%	102	40%
≥ 3	35	11%	46	14%	25	11%	35	14%
missing	0		0		0		0	
Age at first full-term birth among parous women								
nulliparous	39		33		34		25	
< 20	29	11%	30	10%	23	11%	25	11%
20-24	135	49%	156	53%	97	48%	119	52%
25-29	80	29%	74	25%	58	29%	58	25%
≥ 30	31	11%	37	12%	23	11%	26	11%
missing	0		0		0		0	
Menopausal status								
pre-menopausal	292	93%	305	92%	220	94%	233	92%
post-menopausal	22	7%	25	8%	15	6%	20	8%
missing	0		0		0		0	
Family history of breast cancer in first-degree relatives								
no	286	91%	312	95%	213	91%	240	95%
yes	28	9%	18	5%	22	9%	13	5%
missing	0		0		0		0	

Table 1. (continued)

	Eligible cases (N=314)		Cases analyzed for skewing (N=235)	
	N	%	N	%
Tumor Characteristics				
Histologic subtype				
ductal	205	65%	155	66%
lobular	43	14%	33	14%
other	66	21%	47	20%
Lymph node status				
negative	189	61%	140	61%
1-3	78	25%	61	26%
4+	42	14%	30	13%
missing	5		4	
Received chemotherapy before blood collection				
no	302	96%	227	97%
yes	12	4%	8	3%
Estrogen Receptor				
negative	88	34%	61	32%
positive	172	66%	130	68%
missing	54		44	
Progesterone Receptor				
negative	168	65%	126	66%
positive	92	35%	65	34%
missing	54		44	

Table 2. Association between breast cancer case-control status and measures of skewed X-chromosome inactivation.

	All subjects (n=488)							Pre-menopausal, no chemo, <50 years (n=360)[2]						
	Controls (N=253)		Cases (N=235)					Controls (N=194)		Cases (N=166)				
Adjusted % skewing[1]	N	%	N	%	OR[3]	95%CI	*P* value	N	%	N	%	OR[3]	95%CI	*P* value
normal (<50)	157	62.1	147	62.6	Reference (1.0)			126	64.9	106	63.9	Reference (1.0)		
partial (50-74)	66	26.1	53	22.6				46	23.7	37	22.3			
skewed (>75)	30	11.9	35	14.9	1.2	(0.7 - 2.1)		22	11.3	23	13.9	1.2	(0.6 - 2.3)	
Non-parametric tests														
Wilcoxon rank-sum							0.4							0.3
Median							0.8							0.3
Unadjusted skewing measure[4]														
normal	230	90.9	211	89.8	Reference (1.0)			177	91.2	152	91.6	Reference (1.0)		
skewed (>.9, <.1)	23	9.1	24	10.2	1.1	(0.6 - 2.0)		17	8.8	14	8.4	0.9	(0.4 - 2.0)	
Non-parametric tests														
Wilcoxon rank-sum							0.7							0.7
Median							0.9							0.8

1 Absolute value of the percent change in proportion of signal from the shorter allele (allele 1) in the restriction-enzyme digested sample compared to the undigested proportion.

2 Includes only pre-menopausal women under age 50 with no history chemotherapy before blood collection.

3 Odds ratio for showing skewed X-chromosome inactivation (compared to the combined group of Normal and Partial), adjusted for age, age at menarche, number of full-term births, age at first full-term birth, menopausal status (all subjects group only), and first-degree family history of breast cancer.

4 Proportion of signal from the tallest allele in the restriction-enzyme digested sample only.

Table 3. Characteristics of two prior studies of X chromosome inactivation and cancer and the current study.

Setting	Subject characteristics/ number informative	Age range	Family History	Assay[a]	Results
U.S. University Hospital Obstetrics/Gynecology Department (Univ. of Iowa)[4]	Invasive epithelial ovarian cancer (n=174), unrelated controls (n=45), matching not specified	Not fully specified, mean approx. 57	Unknown selection for family history, 11 *BRCA1* mutation carriers	*AR*, ^{32}P-labeled DNA analyzed with acrylamide gels; skewed if visually inspected peak ratio in digested sample $\geq$ 3	53% of cases skewed, 33% of controls skewed, OR = 2.6
Two Norwegian Hospitals[5]	Consecutive series of breast cancer cases from 1984-1994(n=40, diagnosed ages 27-45), controls mostly blood donors (n=90, age 19-45)	27-90, median 60	Unselected for family history	*AR*, fluor-labeled DNA analyzed on ABI 373; skewed if 90% or more of signal was from one allele in digested sample	13% of cases skewed, 1% of controls skewed, OR = 13
Subset of a population-based study in two cities in Poland (Current Study)	Rapidly ascertained cases (n=166, diagnosed < 50), randomly selected population controls (n=194, age <50), matched on residence and age.	25-65, 92% between 35-54	Unselected for family history	*AR*, fluor-labeled DNA analyzed on ABI 3730; skewed if 75% or more of signal was from one allele in digested sample, adjusted to signal in undigested sample	14% of cases skewed, 11% of controls skewed, OR = 1.2

[a] All three studies used the same Androgen Receptor (*AR*) locus[9], but with slightly different platforms\conditions, as outlined in the table.

Figure 1A. Distribution of adjusted skewing measure according to case-control status

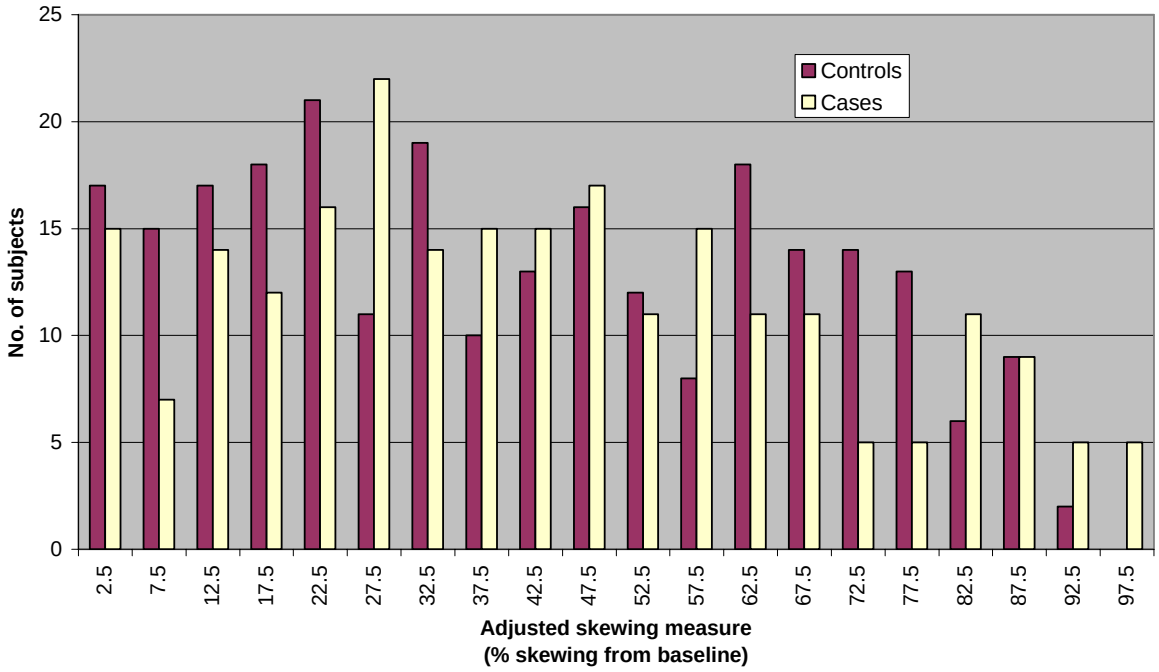

Figure 1B. Distribution of unadjusted skewing measure according to case-control status